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Regulatory effects of fibroblast growth factor-8 and tumor necrosis factor-α on osteoblast marker expression induced by bone morphogenetic protein-2

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ABSTRACT

BMP induces osteoblast differentiation, whereas a key proinflammatory cytokine, TNF-α, causes inflammatory bone damage shown in rheumatoid arthritis. FGF molecules are known to be involved in bone homeostasis. However, the effects of FGF-8 on osteoblast differentiation and the interaction between FGF-8, BMPs and TNF-α have yet to be clarified. Here we investigated the effects of FGF-8 in relation to TNF-α actions on BMP-2-induced osteoblast marker expression using myoblast cell line C2C12, osteoblast precursor cell line MC3T3-E1 and rat calvarial osteoblasts. It was revealed that FGF-8 inhibited BMP-2-induced expression of osteoblast differentiation markers, including Runx2, osteocalcin, alkaline phosphatase, type-1 collagen and osterix, in a concentration-dependent manner. The inhibitory effects of FGF-8 on BMP-induced osteoblast differentiation and Smad1/5/8 activation were enhanced in the presence of TNF-α action. FGF-8 also inhibited BMP-2-induced expression of Wnt5a, which activates a non-canonical Wnt signaling pathway. FGF-8 had no significant influence on the expression levels of TNF receptors, while FGF-8 suppressed the expression of inhibitory Smad6 and Smad7, suggesting a possible feedback activity through FGF to BMP receptor (BMPR) signaling. Of note, inhibition of ERK activity and FGF receptor (FGFR)-dependent protein kinase, but not JNK or NFκB pathway, suppressed the FGF-8 actions on BMP-induced osteoblast differentiation. FGF-8 was revealed to suppress BMP-induced osteoblast differentiation through the ERK pathway and the effects were enhanced by TNF-α. Given the finding that FGF-8 expression was increased in synovial tissues of rheumatoid arthritis, the functional interaction between FGFR and BMPR signaling may be involved in the development process of inflammatory bone damage.

1. Introduction

Bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily, have various activities including cell proliferation, differentiation [1] and endocrine regulation [2]. BMP activity is critical for the differentiation of mesenchymal stem cells into chondrocytes and/or osteoblasts [3].

Cooperative activities of osteoblasts and osteoclasts are crucial for maintenance of bone remodeling. Disruption of the functional balance between these cells leads to impaired bone formation and abnormal bone loss [4]. In rheumatoid arthritis (RA), increased levels and activities of proinflammatory cytokines such as tumor necrosis factor (TNF)-α result in joint and/or bone destruction. A

Abbreviations: ALK, activin receptor-like kinase; ActRII, activin type-II receptor; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; MAPK, mitogen-activated protein kinase; OA, osteoarthritis; RA, rheumatoid arthritis; RANK, receptor activator of NFκB; Runx2, runt-related transcription factor 2; SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; TNFR, TNF receptor.

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paradigm shift in the treatment of RA has been brought about by the clinical application of TNF-α inhibitors [5,6]. TNF-α is known to be a stimulator of osteoclast differentiation. NFκB, one of main signal pathways activated by TNF-α, is essential for RANK- and cytokine-mediated osteoclastogenesis [7]. Moreover, TNF-α not only activates osteoclasts but also inhibits osteoblast maturation [4].

The fibroblast growth factor (FGF) family is composed of 25 related proteins that are involved in many biological processes including cell growth, migration, differentiation and survival. Mutations of FGF receptors cause several genetic skeletal dysmorphic syndromes, suggesting that FGF signaling is essential for bone metabolism [8,9]. FGF-2, -8 and -18 have been implicated as pertinent contributing factors in bone and cartilage homeostasis [10]. In the process of embryonic development, BMP and FGF signals interact to regulate cell differentiation in several organs. For instance, FGF-2 enhances the BMP-4 signal by suppressing the expression of a BMP antagonist, noggin, during skull development [11].

FGF-8 was originally cloned from a conditioned medium of an androgen-dependent mouse mammary carcinoma cell line [12]. In embryonic development, FGF-8 is known to regulate gastrulation and other steps of development [13]. In neurological development, FGF-8 is involved in the isthmic organizing activity and stabilizes or changes the expression of transcription factors in the mid/hindbrain region [14]. It was also reported that FGF-8 plays a regulatory role in cardiovascular development [15], limb development and cranial formation [16]. FGF-8 is expressed in the osteoblast compartment of calvarial bone, mandible, cortical bone, and growth plates of developing long bones in later embryonic development [17,18].

Nevertheless, the detailed mechanism underlying the action of FGF-8 in osteoblast differentiation remains to be elucidated. Previous reports indicated different actions of FGF-8 in the proliferation and differentiation phases of osteoblasts [19]. Of interest, FGF-8 was reported to enhance degradation of the extracellular matrix in osteoarthritis (OA) model rats [20]. There are also some reports suggesting involvement of FGFs, BMPs and inflammatory cytokines in both RA and OA [21,22]. However, the interaction between FGF-8 and BMPs in the process of osteoblast differentiation and the relationship between FGF-8 and proinflammatory cytokines in BMP-induced osteogenesis have not been determined.

In the present study, we investigated the functional crosstalk between FGF-8 and TNF-α receptor signaling, which acts against BMP-2-induced osteoblast differentiation. The results obtained in this study may provide a new clue for understanding and preventing the progress of inflammatory bone damages.

2. Methods

2.1. Reagents and supplies

Recombinant human BMP-2 and mouse FGF-8b were purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant human TNF-α was obtained from PeproTech EC Ltd. (London, UK). The MEK inhibitor U0126 was from Promega Corp. (Madison, WI), JNK inhibitor SP600125 was from Biomol Lab., Inc. (Plymouth Meeting, PA), inhibitor of the tyrosine kinase activity FGF receptor SU5402, and NFκB activation inhibitor IV were from Calbiochem (San Diego, CA). Total RNAs purified from the synovium resected from RA and normal patients were purchased from OriGene Technologies, Inc. (Rockville, MD).

2.2. Cell culture

The mouse myoblast cell line C2C12 and osteoblast precursor cell line MC3T3-E1 were cultured in high-glucose DMEM and MEM Alpha, respectively, at 37 °C under a humid atmosphere with 5% CO_2. Each medium was supplemented with 10% FCS and penicillin–streptomycin solution. Rat calvarial osteoblast cells, dissociated from Sprague-Dawley rat embryos, were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and cultured in high-glucose DMEM supplemented with 10% FCS and growth SingleQuots™ kit (Lonza Walkersville) following the manufacturer's protocol.

2.3. RNA extraction and quantitative real-time PCR analysis

To prepare total cellular RNA, cells were cultured in a 12-well plate and treated with the indicated concentrations of TNF-α and BMP-2 in combination with FGF-8, U0126, SP600125, SU5402 or NFκB inhibitor in serum-free DMEM. After 24- or 48 h culture, the medium was removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). The extracted RNA (1.0 μg) was subjected to RT reaction using the First-Strand cDNA synthesis system® (Invitrogen Corp.). Primer pairs were selected from different exons of the corresponding genes as follows: Wnt5a, 1210–1230 and 1365–1385 (from NM_009524); and FGF-8, 46–66 and 159–179 (from U36223). Primer pairs for osteocalcin, osterix, alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), type-1 collagen (collagen-1), Id-1, TNF receptor 1 (TNFR1) and TNFR2, BMP type-I and type-II receptors, Smads and a housekeeping gene ribosomal protein L19 (RPL19) were selected as we reported [23,24] (Table 1). Real-time PCR was performed using the StepOnePlus® real-time PCR system (Applied Biosystems, Foster City, CA) under optimized annealing conditions, according to the manufacturer's protocol with the following profile: 40 cycles each at 95 °C for 3 s and 58–62 °C for 30 s. Ct values were calculated using StepOnePlus™ system software (Applied Biosystems). The relative expression of each mRNA was calculated by the ΔCt Method, in which ΔCt is the value obtained by subtracting the Ct value of RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to RLP19 mRNA was expressed as $2^{-(\Delta Ct)}$. Data are expressed as the ratio of target mRNA to RPL19 mRNA.

2.4. Western immunoblot analysis

After preculture, the medium was replaced with fresh serum-free medium, and after 16 h culture, cells were treated with the indicated concentrations of BMP-2, TNF-α and FGF-8. Following stimulation with growth factors for 15–60 min, the cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM sodium fluoride, 2% sodium dodecyl sulfate, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-phospho-Smad1/5/8, anti-Smad1, anti-phospho- and total-ERK1/2, anti-phospho- and total-P38, anti-phospho- and total-SAPK/JNK, anti-phospho- and total-NFκB-p65, anti-phospho-FGFRs (Cell Signaling Technology, Inc., Beverly, MA), anti-FGFR3 and FGFR4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-actin antibodies (Sigma–Aldrich Co., Ltd., MO). The relative integrated density of each protein band was digitized by NIH image J 1.34 s or by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE).

2.5. Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The differences between groups were analyzed for statistical significance using ANOVA with Fisher's PLSD test or unpaired *t*-test, when appropriate, to determine differences (JMP 9.0 software package,